(54) Title: DIAGNOSTIC METHOD FOR ALZHEIMER'S DISEASE

(57) Abstract

The present invention is directed to methods and kits for diagnosing, subtyping and monitoring Alzheimer's disease. The invention is based, in part, on the discovery that proteolytic fragments of the amino and carboxy terminal amino acid residues of *tau*-proteins are released from the neurofibrillary tangles associated with the disease and can be detected in body fluids outside the brain. The *tau*-proteins will be purified or chemically synthesized and peptide fragments of the amino terminal and carboxy terminal regions will be obtained proteolytically or synthesized chemically and will be used in generating *tau* specific antibodies for use in diagnostic kits for the detection of Alzheimer's disease. These diagnostic kits will be used in screening the body fluids of individuals for the presence of *tau*-peptide fragments. Alternatively, the *tau*-peptides themselves may be used in diagnostic kits for screening the body fluids of individuals for the presence of circulating autoantibodies.

& Meyer, 1979, *Medical Neurology*, pp. 175-179
MacMillan Publishing Co.). Alzheimer's disease
afflicts an estimated 4 million human beings in the
United States alone at a cost of 35 billion dollars a
5   year (Hay & Ernst, 1987, *Am. J. Public Health*,
77:1169-1175). It is found in 10% of the population
over the age of 65 and 47% of the population over the
age of 85 (Evans *et al.*, 1989, *JAMA*, 262:2551-2556).
In addition, the disease is found at much lower levels
10   in the younger age groups, usually beginning at about
30 years of age and even rarely in late childhood
(Adams & Victor, 1977, *Principles of Neurology*, pp.
401-407).
        The etiology of Alzheimer's disease is
15   unknown. Evidence for a genetic contribution comes
from several important observations such as the
familial incidence, pedigree analysis, monozygotic and
dizygotic twin studies and the association of the
disease with Down's syndrome (for review see
20   Baraitser, 1990, *The Genetics of Neurological
Disorders*, 2nd edition, pp. 85-88). Nevertheless,
this evidence is far from definitive and it is clear
that one or more other factors are also required.
Elevated concentrations of aluminum have been found in
25   the brains of some patients dying with Alzheimer's
disease (Crapper *et al.*, 1976, *Brain*, 99:67-80) and
one case report has documented markedly elevated
levels of manganese in the tissues of a patient with
Alzheimer's disease (Banta & Markesberg, 1977,
30   *Neurology*, 27:213-216), which has led to the
suggestion that high levels of these metals may be
neurotoxic and lead to the development of Alzheimer's
disease. It was interesting that the aluminum ions
were found to be associated mainly with the nuclear
35   chromatin in brain regions most likely to display
neurofibrillary tangles in Alzheimer's disease.
However, from a statistical point of view the absolute

differences found for the aluminum levels between
normal and Alzheimer brains were far from convincing.
It has recently been suggested that defects in the
transcriptional splicing of mRNA coding for the tau
5 complex of microtubule associated proteins occur (for
review see Kosik, 1990, *Curr. Opinion Cell Biol.*,
2:101-104) and/or that inappropriate phosphorylation
of these proteins exists (Grundke-Igbak *et al.*, 1986,
Proc. Natl. Acad. Sci. USA, 83:4913-4917; Wolozin &
10 Davies, 1987, *Ann. Neurol.* 22:521-526; Hyman *et al.*,
1988, *Ann. Neurol.*, 23:371-379; Bancher *et al.*, 1989,
Brain Res., 477:90-99). Furthermore, reduction in the
enzymes involved in the synthesis of acetylcholine has
led to the view of Alzheimer's disease as a
15 cholinergic system failure (Danes & Moloney, 1976,
Lancet, ii:1403-14). However, even if cholinergic
neurons are most at risk in Alzheimer's disease, it
appears likely that these reductions in enzyme
activity are secondary to the degenerative process
20 itself rather than causally related.

2.1 THE DIAGNOSIS OF ALZHEIMER'S DISEASE

The diagnosis of Alzheimer's disease at
autopsy is somewhat time-consuming but is definitive.
25 The gross pathological changes are found in the brain,
which is underweight, and shows generalized atrophy of
both the gray and white matter of the cerebral cortex,
particularly in the temporal and frontal lobes, with
widening of the sulci and narrowing of the gyri as
30 well as compensatory symmetrical dilation of the third
and lateral ventricles (Adams & Victor, 1977,
Principles of Neurology, pp. 401-407; Merritt, 1979, *A
Textbook of Neurology*, 6th edition, Lea & Febiger,
Philadelphia, pp. 484-489). The histological changes
35 in the brain are definitive. The most common lesion
is the neurofibrillary tangle (Kidd, 1963, *Nature*,
197:192-193; Kidd, 1964, Brain 87:307-320), which

consists of a tangled mass of paired helical filaments and occasional straight filaments located in the cytoplasm of affected neurons (Oyanagei, 1979, *Adv. Neurol. Sci.*, 18:77-88 (in Japanese) as quoted by

5 Grundke-Iqbal et al., 1985, *Acta Neuropathol.*, 66:52-61). The other characteristic histological lesions are Hirano bodies within nerve cells of certain brain regions, neuritic dystrophy and amyloid angiopathy. In addition, there are prominent argentophilic senile

10 neuritic plaques composed of enlarged, degenerating axonal endings, containing degraded cellular organelles and some paired helical filaments, that surround a core of mainly extracellular amyloid (Merritt, 1979, *A Textbook of Neurology*, 6th edition,

15 Lea & Febiger, Philadelphia, pp. 484-489). Small amounts of these histological changes are found also in the brains of almost all very old humans at autopsy, even if they showed no symptoms of Alzheimer's disease before death (Tomlinson &

20 Henderson, 1976, *Neurobiology of Aging*, p. 183, Terry & Gershon (eds.), Raven, New York; Ball, 1976, *Neuropathol. Appl. Neurobiol.*, 2:395; Ball, 1977, *Acta Neuropathol.*, 37:111-118). However, the number and total amount of these histological changes in brain

25 has been found to correlate well with the severity of the clinical signs and symptoms of Alzheimer's disease (Kidd, 1963, *Nature*, 197:192-193; Terry et al., 1964, *Am. J. Pathol.*, 44:269-297; Roth et al., 1966, *Nature*, 209:109-110; Blessed et al., 1968, *Br. J. Psychiat.*,

30 114:797-881; Tomlinson et al., 1970, *J. Neurol. Sci.*, 11:205-242; Wisniewski, 1976, *J. Neurol. Sci.*, 27:173-181).

The diagnosis of Alzheimer's disease during life is more difficult than at autopsy since it

35 depends upon an inexact clinical evaluation. In the middle stages and particularly in the earlier stages of the disease, the diagnosis is a matter of clinical

judgement and success is markedly dependent upon the
experience and clinical acumen of the attending
physician or consultant. In the late stages of the
disease however, clinical diagnosis is relatively
5 straight-forward even if it is not completely
unequivocal. In all cases, a wide variety of other
diseases, presenting at least partially overlapping
clinical pictures, must be positively ruled out before
a diagnosis of Alzheimer's disease can be made.
10 Usually a patient must be evaluated on a number of
occasions over some period of time in order to
document the deterioration in intellectual ability and
other signs and symptoms before a diagnosis can be
attempted with reasonable confidence. The necessity
15 of repeated evaluation by a professional medical
practitioner before a firm diagnosis can be made is
costly, generates anxiety and can be frustrating to
patients and their families. Furthermore, the
development of an appropriate therapeutic strategy is
20 greatly hampered by these difficulties in the
objective and rapid diagnosis of the disease,
particularly in the early stages where even a simple
arrest of the progress of the disease would leave the
patient with significant intellectual capacity and a
25 reasonable quality of life.

No unequivocal laboratory test specific for
Alzheimer's disease has been reported. European
patent application No. 391714 discloses a method for
the diagnosis of Alzheimer's disease based upon the
30 detection of mature β-amyloid protein, its precursor
protein or a fragment of β-amyloid protein in non-
neural tissue biopsy using immunoassay techniques.
However, this approach is non-specific since it has
been recognized that a wide variety of degenerative
35 diseases, lead to the production and deposition of β-
amyloid protein in almost all human tissues.
Furthermore, even though the gene coding for both the

β-amyloid protein (Goldgaber *et al.*, 1987, *Science,*
235:877-80; Tanzi *et al.*, 1987a, *Science,* 235:880-884)
and the gene for familial Alzheimer's disease (St.
George-Hyslop *et al.*, 1987, *Science,* 325:885-890) have
5   been located on human chromosome 21, it has been shown
that the linkage between the two genes is not very
close (Van Broedkhoven *et al.*, 1987, *Nature,* 329:153-
155; Tanzi *et al.*, 1987b, *Nature,* 329:156-157).   In
addition, it is known that despite some earlier
10 claims, β-amyloid protein forms no part of the paired
helical filaments within neurofibrillary tangles (for
review see Wischik *et al.*, 1989, *Curr. Opinion Cell
Biol.,* 1:115-122).   Therefore, even though β-amyloid
protein is deposited in many tissues of patients with
15 Alzheimer's disease, it has not been definitively
linked to Alzheimer's disease in a manner different to
its association with many other degenerative diseases.
Thus, the diagnostic usefulness of antibodies directed
to mature β-amyloid protein, its precursor or any of
20 its fragments must be considered marginal at best.

2.2   NEUROFIBRILLARY TANGLES AND
ALZHEIMER'S DISEASE

It is now known that the only definitive
25 feature of Alzheimer's disease is the presence of
grossly increased quantities of neurofibrillary
tangles within the affected cortical regions of brain
compared to either normal brain from humans of any age
or to brain from any disease state other than
30 Alzheimer's disease itself.   However, the
neurofibrillary tangles have not heretofore been
exploited for use in a diagnostic or screening assay
for Alzheimer's disease.
                    The progression of Alzheimer's disease is
35 characterized by a loss of cortical substance in the
brain.   This fact has been well documented over many
years by many different researchers.   The most

characteristic lesion in Alzheimer's disease is the presence of paired helical filaments in discrete, randomly interwoven groups or neurofibrillary tangles within affected cortical neurons. In addition, the

5 number and size of these tangles within an affected neuron as well as the total number of tangles and the total amount of constituent tau-protein in an affected brain correlates with the progression and severity of the disease. Therefore, it is believed that as these

10 tangles increase in size and number they must interfere with the physiological function of each cell in which they occur, eventually leading directly to the death and lysis of that cell. Recently, it has been reported that the cytosolic ATP-dependent

15 protease signal protein, ubiquitin, becomes attached to neurofibrillary tangles (Mori et al., 1987, Science, 235:1641-1644). Since ubiquitinated proteins are rapidly degraded, this suggests that the affected cell recognizes the tangles as a foreign structure

20 requiring degradation. It is further believed that this mechanism of cortical neuron death is responsible for the progressive loss of cortical substance and, therefore, the loss of intellectual capacity as well as the appearance of the other signs and symptoms of

25 Alzheimer's disease.

The paired helical filaments are composed of an unknown protein of 90,000 molecular weight (Mr) associated with various tau-protein species. A tightly bound helical core is formed between the

30 90,000 Mr protein and the large middle domain of the tau-protein as well as a region of the C-terminal domain, which contains the imperfect tandem repeat. The structural conformation of the helical core allows the N-terminal and C-terminal domains of the tau-

35 protein to protrude at some angle from the axis of the paired helical filament thereby forming a protease-sensitive coat around the paired helical filaments

(Wischik *et al.*, 1988, *Proc. Natl. Acad. Sci. USA.*, 85:4884-4888). The research groups working in this area are directing their efforts towards characterizing the protease-resistant core of the

5 paired helical filaments in order to understand the nature of the stable interaction and the nature of the modification that occurs to *tau*-proteins in Alzheimer's disease (for reviews, see Wischik *et al.*, 1989, *Curr. Opinion Cell Biol.*, 1:115-122; Kosik,

10 1990, *Curr. Opinion Cell Biol.*, 2:101-104). This orientation of research effort as well as research directed towards the molecular genetics of the *tau*-protein are likely to lead to an understanding of the primary cause of Alzheimer's disease.

15   From the foregoing, it is clear that no one concerned with Alzheimer's disease has realized that its diagnosis ultimately must depend upon the detection and quantitative measurement of the amount of the component substance or substances that

20 constitute these neurofibrillary tangles in victims of the disease. The detection of associated substances that can also be found in other disease states can never be as definitive as the detection of the actual substance building up in affected neurons in

25 Alzheimer's disease causing the eventual death of those cells. What has also not been realized is that the protease stability of the core of the paired helical filaments makes it unlikely that any part of the *tau*-protein or the 90,000 Mr protein present in

30 this stable core will ever be found outside the brain. The slow destruction of these stable cores, following their release by dead neurons, can be conceived to occur only *in situ* and as a consequence of phagocytosis by microglia. The most likely reason for

35 the reluctance or failure to recognize the potential of using components of the neurofibrillary tangles in the diagnosis of Alzheimer's disease relates to the

natural abhorrence that a person would feel if required to biopsy the brain of any living human being even in the last stages of the disease in order to measure these substances. The biopsy procedure is
5 extremely invasive and must leave the person so biopsied less than whole. However, the scientific community has not considered the possibility that biopsy might not be necessary in order to detect the substance or substances in question.

10 In contrast to the relevant literature, the diagnostic approach of the present invention is based on the recognition that the whole of the 200 amino acid N-terminal residues of the various *tau*-proteins as well as some portion of their 50 amino acid most C-
15 terminal residues will be released when cleaved from the filaments by ubiquitin-recognizing proteases or other proteases during degeneration and rupture of affected neurons. The diagnostic assays of the invention are based on the principle that it is these
20 cleaved latter segments of *tau*-proteins that can find their way into body fluids outside the brain and whose detection and quantitation are important for diagnostic purposes. Additional support for this last conclusion comes from the observation that other
25 brain-specific, soluble and protease-sensitive proteins find their way into blood and spinal fluid following head trauma (Phillips *et al*., 1980, *Br. Med. J.*, **281**:777-779; Brayne *et al*., 1982, *Lancet*, **ii**:1308-9) and various other types of neurological disorders
30 (Thompson *et al*., 1980, *J. Neurol. Sci.*, **47**:241-254; Willson *et al*., 1980, *Ann. Clin. Biochem.*, **17**:110-113).

3. SUMMARY OF THE INVENTION
35 The present invention relates to methods and kits for diagnosing, subtyping and monitoring Alzheimer's disease. The invention is based, in part,

on the discovery that proteolytic fragments of the
amino and carboxy terminal amino acid residues of *tau*-
proteins are released from the neurofibrillary tangles
associated with the disease and can be detected in
5 body fluids outside the brain.

The generation and purification of *tau*-
proteins and peptide fragments of the amino terminal
and carboxy terminal regions of *tau*-proteins are
described. These *tau*-proteins and fragments thereof
10 may be used to generate monoclonal antibodies,
polyclonal antibodies or Fab fragments that, in turn,
can be used in immunoassays to detect circulating *tau*-
peptides in suspected individuals. Alternatively, the
tau-peptides themselves may be used in immunoassays to
15 detect circulating autoantibodies in such individuals.

3.1 DEFINITIONS

The term *tau*-protein is defined herein as
any one of the proteins that comprise a single
20 constituent part of the *tau*-complex of proteins.

The *tau*-complex of proteins is defined as
that group of microtubule associated proteins (MAPS)
from vertebrate brain that have the following
characteristics: (a) co-purify with tubulin during the
25 cycle purification of microtubules, particularly in
the absence of glycerol (Asnes & Wilson, 1979, *Anal.
Biochem.*, 98:64-73; Murphy & Hiebsch, 1979, *Anal.
Biochem.*, 96:225-235); (b) separate from tubulin
present in the microtobules obtained by the cycle
30 technique when an ice-cold sample of such a
preparation is chromatographed on a phosphocellulose
column, using 2-(N-morpholino)-ethanesulphonic acid
(MES) buffer to remove tubulin and using MES buffer
containing 1 M NaCl to remove the *tau*-complex of
35 proteins (Weingarten *et al.*, 1975, *Proc. Natl. Acad.
Sci. USA*, 72:1858-1862; Cleveland *et al.*, 1977, *J.
Mol. Biol.*, 116:207-225; Cleveland *et al.*, 1979, *J.

Biol. Chem., 254:12670-12678); (c) are included in a
column of Sepharose 6B when chromatographed on such a
matrix and where they migrate as an aggregated species
not far from the exclusion volume but have individual
5   unaggregated native molecular masses between 10,000
and 100,000 Mr regardless of axial ratio (Cleveland et
al., 1977, *J. Mol. Biol.*, 116:207-225); (d) react with
any of a number of antibodies described in the
literature as "anti-*tau*" or with a commercial antibody
10 described as anti-*tau*; (e) contain within each and
every one of their C-terminal domains an imperfect
tandem repeat of the type described in the literature
(Lewis et al., 1988, *Science*, 242:936-939; Goedert et
al., 1989, *EMBO J.*, 8:393-399; Kanai et al., 1989, *J.
15 Cell Biol.*, 109:1173-1184); and (f) are either heat-
stable or heat-labile as described by Cleveland et
al., 1977, *J. Mol. Biol.*, 116:207-225.

The term "forward sandwich assay" is defined
in its most basic form as an assay wherein the sample
20 to be tested containing the *tau*-peptide reacts with an
anti-*tau*-peptide antibody that has been immobilzed
upon a solid substrate; then a labeled second anti-
tau-peptide antibody is added, which reacts with the
tau-peptide at a different site.

25   The term "reverse sandwich assay" is defined
in its most basic form as an assay wherein the labeled
anti-*tau*-peptide antibody and sample to be tested
containing the *tau*-peptide are first incubated
together and then added to a second unlabeled anti-
30 *tau*-peptide antibody that had previously been
immobilized upon a solid substrate as in the "forward
sandwich assay". Both the labeled and the unlabeled
anti-*tau*-peptide must react with the *tau*-peptide at a
different site.
35

4. DESCRIPTION OF THE FIGURE

Figure 1 shows the complete nucleotide and amino acid sequences of one form of human *tau-protein*, reproduced from Goedert *et al.*, 1988, *Proc. Natl.*

5 *Acad. Sci. USA*, 85:4051-4055.

5. DETAILED DESCRIPTION OF THE INVENTION

The present invention results from the unexpected discovery of several physiological functions for individual proteins in the *tau-complex*

10 of proteins that have not heretofore been described and are in addition to the well recognized function of promoting the polymerization of microtubules and/or their stability. This discovery has permitted the development of assays for the individual proteins in

15 the *tau-complex* of proteins and their subsequent purification with retention of their native configuration and physiological activity. The invention is based, in part, on the discovery that in patients suffering from Alzheimer's disease there is

20 proteolytic fragmentation of the *tau-complex* of proteins which occurs independently of phosphorylation, such that only the N-terminal domain comprising up to not more than the 200 most N-terminal amino acids or less and the C-terminal domain

25 comprising up to only the 50 most C-terminal amino acids or less of *tau* will be released from the paired helical filaments, resident in the neurofibrillary tangle or free in the cytoplasm of affected neurons,

30 and make their way into spinal fluid and blood.

This invention provides the first demonstration that any part of the tau-proteins of brain in victims of Alzheimer's disease are also present in body fluids outside the brain itself in

35 concentrations greatly exceeding that found in normal humans that are not victims of Alzheimer's disease. This is achieved by examining a sample of body fluid,

- 13 -

preferably blood or spinal fluid, and detecting the
presence and quantity of any of the *tau*-proteins or
fragments thereof. The detection and quantitative
measurement of any of these *tau*-proteins or fragments
5   thereof in body fluids in accordance with this
invention is useful in confirming a clinical diagnosis
of Alzheimer's disease in affected patients and in
following the course of the disease. The invention is
also useful in monitoring the disease during and
10   subsequent to a period of treatment with agents that
are being tested for their ability to stabilize,
decrease or prevent the release of *tau*-proteins or
fragments thereof from the brain.

   Alternatively, the invention is directed to
15   the production of *tau*-peptides for use in detecting
circulating anti-*tau*-peptide autoantibodies in body
fluids outside the brain in victims of Alzheimer's
disease. The detection of circulating anti-*tau*-
peptide autoantibodies in accordance with this
20   invention is useful in confirming a clinical diagnosis
of Alzheimers disease in affected patients and in
following the course of the disease.

   For purposes of description only, the
invention will be described in terms of: (a)
25   generating *tau*-peptides; (b) generating antibodies
that define the *tau*-peptide; and (c) diagnostic assays
and kits for diagnosing subtyping or monitoring
Alzheimer's disease.

30   **5.1 PRODUCTION OF *TAU*-PEPTIDES**
  5.1.1 ENZYMATIC CLEAVAGE OF PURIFIED *TAU*-PROTEINS
   The production of *tau*-peptides may be
accomplished by isolating the paired helical filaments
from human brains of patients who died with Alzheimers
35   disease and purifying the helical complex containing
the *tau*-protein and the 90,000 Mr protein (*see* Section
6, *infra*). The *tau*-protein is associated with the

90,000 Mr protein such that the 200 most amino terminal residues and the 50 most carboxy terminal residues protrude from the helical core of the complex thereby rendering those fragments susceptible to
5 proteolytic enzymes.

The cleavage of the tau-protein into two large fragments of amino acid residues 1 to 151 and 154 to 352 and the dipeptide, Ser-Arg, may be accomplished using either one of the arginine-specific
10 proteolytic enzymes such as thrombin (EC 3.4.13) as described recently (Joly et al., 1989, J. Cell Biol., 109:2289-2294) or clostridiopeptidase B (clostripain) (EC 3.4.4.20). The preferred embodiment uses clostripain because the enzyme is a sulphydryl enzyme,
15 requiring calcium ions for full activity. Consequently, its activity can be terminated conveniently by the addition of excess iodoacetamide and ethylene glycol-bis(β-aminoethyl ether) N,N, N',N'-tetraacetic acid (EGTA). In the case of either
20 protease the concentration of tau-protein is adjusted to 100 μg/ml before beginning the digestion. Purified clostripain is used at a concentration of 4 international units/ml in the following buffer (50 mM phosphate buffer pH 7.7, 1 mM β-mercaptoethanol, and 1
25 mM calcium acetate). Soybean trypsin inhibitor (clostripain/inhibitor molar ratio = 10/1) is added in order to eliminate any small residual trypsin activity in commercially available preparations of the enzyme. The cleavage reaction is conducted at 37°C and is
30 terminated by the addition of a sufficient volume of a 100 mM stock solution EGTA, pH 7.7, containing 100 mM iodoacetimide to give a final concentration of 2 mM EGTA in the protease reaction mixture. The exact time required for proteolysis varies with each tau-protein
35 and markedly depends upon the batch of clostripain used. Therefore, the optimum time for a single cleavage must be determined for each combination of

clostripain batch and *tau*-protein used. The time course of the cleavage reaction is determined by conducting the reaction as described above, but on a 20 μl volume and assaying the cleavage reaction by

5 subjecting samples at various times to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) according to the method of Laemmli, (*Nature*, 1970, 227:680-684) as modified by Studier, *Sci.*, 176:367-376 (1972) using a 10% (w/v) polyacrylamide resolving gel

10 followed by Serva blue staining and destaining in the usual manner of anyone familiar with the art.

The two large *tau*-peptide fragments resulting from either thrombin or clostripain proteolysis may be further cleaved by digestion with

15 trypsin, which cleaves on the carboxy terminus of lysine or arginine residues. See for example, the techniques described in *Current Protocols in Molecular Biology*, Ausubel et al., Green Publish. Assoc. & Wiley Interscience, Ch. 10.

20 The sequence of *tau*-peptides derived from proteolytic digestion may be identified using the Edman degradation method of protein sequencing. This method sequentially removes one amino acid residue at a time from the amino terminal end of a peptide for

25 subsequent sequence identification by chromatographic procedures. See for example, the techniques described in Konigsberg and Steinman, 1977, *Strategy and Methods of Sequence Analysis*, in Neurath and Hill (eds.), The Proteins (3rd ed.) Vol. 3, pp. 1-178, Academic Press.

30 In addition, sequence analysis of *tau*-peptides may be accelerated by using an automated liquid phase amino acid sequenator following recently described techniques (Hewick et al., 1981, *J. Biol. Chem.*, 256:7990-7997; Stein and Undefriend, 1984, *Analy.*

35 *Chem.*, 136:7-23), thereby allowing for the analysis of picomolar quantities of *tau*-peptides containing up to 50 amino acid residues in length.

5.1.2 CHEMICAL SYNTHESIS OF TAU-PEPTIDES

Tau-peptides can also be produced by chemical synthesis of the amino acid sequence of a tau-protein (Goedert et al., 1988, *Proc. Natl. Acad.*
5 *Sci. USA*, 85:4051-4055), as predicted from the cloning and sequencing of a cDNA coding for a *tau-protein*. This *tau-protein* sequence information may be utilized to predict the appropriate amino and carboxy terminal tau-peptides to be chemically synthesized using
10 standard peptide synthesis methods known in the art. These methods include a solid-phase method devised by R. Bruce Merrifield, (Erickson and Merrifield, "Solid-Phase Peptide Synthesis", in *The Proteins*, Volume 2, H. Neurath & R. Hill (eds.) Academic Press, I:
15 York pp. 255-257; Merrifield, 1986, "Solid ph synthesis", *Science*, 242:341-347). In the sc method, amino acids are added stepwise to a ç peptide chain that is linked to an insoluble such as polystyrene beads. A major advantag
20 method is that the desired product at each s bound to beads that can be rapidly filtered and thus the need to purify intermediates is obviated. All of the reactions are carried out in a single vessel, which eliminates losses due to repeated
25 transfers of products. This solid phase method of chemical peptide synthesis can readily be automated making it feasible to routinely synthesize peptides containing about 50 residues in good yield and purity (Stewart and Young, 1984, *Solid Phase Peptide*
30 *Synthesis*, 2nd ed., Pierce Chemical Co.; Tam et al., 1983, *J. Am. Chem. Soc.*, 105:6442). For example, tau-peptides corresponding to amino acid residues 1 to 30 and 331 to 352 as depicted in Figure 1 could be synthesized.
35

5.1.3 CLONING AND EXPRESSION OF RECOMBINANT TAU-PROTEINS AND/OR TAU-PEPTIDES

The production of *tau*-peptides can further
be achieved by recombinant DNA technology. For
example, appropriate *tau* nucleotide coding sequences
5   may be synthesized, cloned and expressed in
appropriate host cells. Since the DNA sequence
encoding for a *tau*-protein is known (Goeddert *et al.*,
1988, *Proc. Natl. Acad. Sci.*, USA 85:4051-4055), DNA
probes may be synthesized by standard methods known in
10 the art to screen cDNA libraries prepared from brain
tissue of Alzheimer's disease patients for the
specific tau-protein cDNA's. These DNA probes can
further be used to isolate the entire family of *tau*-
protein genes from these cDNA libraries using methods
15 which are well known to those skilled in the art.
See, for example, the techniques described in Maniatis
et al., 1982, *Molecular Cloning A Laboratory Manual*,
Cold Spring Harbor Laboratory, N.Y., Chapter 7.

The polymerase chain reaction (PCR)
20 technique can be utilized to amplify the individual
members of the *tau* family for subsequent cloning and
expression of *tau*-protein cDNAs (*e.g.*, see U.S. patent
numbers 4,683,202; 4,683,195; 4,889,818; Gyllensten *et
al.*, 1988, *Proc. Nat'l Acad. Sci. USA*, **85:**7652-7656;
25 Ochman *et al.*, 1988, *Genetics*, **120:**621-623; Triglia *et
al.*, 1988, *Nucl. Acids. Res.*, **16:**8156; Frohman *et al.*,
1988, *Proc. Nat'l Acad. Sci. USA*, **85:**8998-9002; Loh *et
al.*, 1989, *Science*, **243:**217-220).

Methods which are well known to those
30 skilled in the art can be used to construct expression
vectors containing *tau*-proteins or fragments thereof
coding sequences and appropriate
transcriptional/translational control signals. These
35 methods include *in vitro* recombinant DNA techniques,
synthetic techniques and *in vivo* recombination/genetic
recombination. See, for example, the techniques

described in Maniatis et al., 1982, *Molecular Cloning A Laboratory Manual*, Cold Spring Harbor Laboratory, N.Y., Chapter 12.

5   A variety of host-expression vector systems may be utilized to express *tau*-proteins or fragments thereof. These include but are not limited to microorganisms such as bacteria transformed with recombinant bacteriophage DNA, plasmid DNA or cosmid DNA expression vectors containing a coding sequence

10   for a *tau*-protein or fragment thereof; yeast transformed with recombinant yeast expression vectors containing a coding sequence for a *tau*-protein or fragment thereof; insect cell systems infected with recombinant virus expression vectors (*e.g.*,

15   baculovirus) containing a coding sequence for a *tau*-protein or fragment thereof; or animal cell systems infected with recombinant virus expression vectors (*e.g.*, adenovirus, vaccinia virus) containing a coding sequence for a *tau*-protein or fragment thereof.

20   The expression elements of these vectors vary in their strength and specificities. Depending on the host/vector system utilized, any of a number of suitable transcription and translation elements, including constitutive and inducible promoters, may be

25   used in the expression vector. For example, when cloning in bacterial systems, inducible promoters such as pL of bacteriophage λ, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the like may be used; when cloning in insect cell systems, promoters such as the

30   baculovirus polyhedrin promoter may be used; when cloning in mammalian cell systems, promoters such as the adenovirus late promoter or the vaccinia virus 7.5K promoter may be used. Promoters produced by recombinant DNA or synthetic techniques may also be

35   used to provide for transcription of the inserted coding sequence for a *tau*-protein or fragment thereof.

In yeast, a number of vectors containing
constitutive or inducible promoters may be used. For
a review see, Current Protocols in Molecular Biology,
Vol. 2, 1988, Ed. Ausubel et al., Greene Publish.
5 Assoc. & Wiley Interscience Ch. 13; Grant et al.,
1987, Expression and Secretion Vectors for Yeast, in
Methods in Enzymology, Eds. Wu & Grossman, 1987, Acad.
Press, N.Y., Vol. 153, pp. 516-544; Glover, 1986, DNA
Cloning, Vol. II, IRL Press, Wash., D.C. Ch.3; and
10 Bitter, 1987, Heterologous Gene Expression in Yeast,
Methods in Enzymology, Eds. Berger & Kimmel, Acad.
Press, N.Y., Vol. 152, pp. 673-684; and The Molecular
Biology of the Yeast Saccharomyces, 1982, Eds.
Strathern et al., Cold Spring Harbor Press, Vols. I
15 and II. For complementation assays in yeast, cDNAs
for tau-proteins or fragments thereof may be cloned
into yeast episomal plasmids (YEp) which replicate
autonomously in yeast due to the presence of the yeast
2μ circle. The tau-protein or fragment thereof
20 sequence may be cloned behind either a constitutive
yeast promoter such as ADH or LEU2 or an inducible
promoter such as GAL (Cloning in Yeast, Chpt. 3, R.
Rothstein In; DNA Cloning Vol. 11, A Practical
Approach, Ed. DM Glover, 1986, IRL Press, Wash.,
25 D.C.). Constructs may contain the 5' and 3' non-
translated regions of a cognate tau-protein mRNA or
those corresponding to a yeast gene. YEp plasmids
transform at high efficiency and the plasmids are
extremely stable. Alternatively vectors may be used
30 which promote integration of foreign DNA sequences
into the yeast chromosome.
        A particularly good expression system which
could be used to express tau-proteins or fragments
thereof is an insect system. In one such system,
35 Autographa californica nuclear polyhedrosis virus
(AcNPV) is used as a vector to express foreign genes.
The virus grows in Spodoptera frugiperda cells. The

tau-protein or fragment thereof coding sequence may be cloned into non-essential regions (for example the polyhedrin gene) of the virus and placed under control of an AcNPV promoter (for example the polyhedrin

5 promoter). Successful insertion of the polyhedrin gene results in production of non-occluded recombinant virus (*i.e.*, virus lacking the proteinaceous coat coded for by the polyhedrin gene). These recombinant viruses are then used to infect *Spodoptera frugiperda*

10 cells in which the inserted gene is expressed. (*e.g.*, see Smith *et al.*, 1983, *J. Biol.*, 46:586; Smith, U.S. Patent No. 4,215,051).

In cases where an adenovirus is used as an expression vector, the *tau*-protein or fragment thereof

15 coding sequence may be ligated to an adenovirus transcription/translation control complex, *e.g.*, the late promoter and tripartite leader sequence. This chimeric gene may then be inserted in the adenovirus genome by *in vivo* or *in vitro* recombination.

20 Insertion in a non-essential region of the viral genome (*e.g.*, region E1 or E3) will result in a recombinant virus that is viable and capable of expressing the *tau*-protein of fragment thereof in infected hosts. (*e.g.*, See Logan & Shenk, 1984, *Proc.*

25 *Natl. Acad. Sci.*, (USA) 81:3655-3659). Alternatively, the vaccinia 7.5K promoter may be used. (*e.g.*, see Mackett *et al.*, 1982, *Proc. Natl. Acad. Sci.*, (USA) 79:7415-7419; Mackett *et al.*, 1984, *J. Virol.*, 49:857-864; Panicali *et al.*, 1982, *Proc. Natl. Acad. Sci.*,

30 79:4927-4931).

Specific initiation signals may also be required for efficient translation of the inserted tau-protein or fragment thereof coding sequences. These signals include the ATG initiation codon and

35 adjacent sequences. In cases where the entire *tau*-protein genome, including its own initiation codon and adjacent sequences, are inserted into the appropriate

expression vectors, no additional translational
control signals may be needed. However, in cases
where only a portion of the tau-protein coding
sequence is inserted, exogenous translational control
5 signals, including the ATG initiation codon, must be
provided. Furthermore, the initiation codon must be
in phase with the reading frame of the tau-protein or
fragment thereof coding sequence to ensure translation
of the entire insert. These exogenous translational
10 control signals and initiation codons can be of a
variety of origins, both natural and synthetic. The
efficiency of expression may be enhanced by the
inclusion of appropriate transcription enhancer
elements, transcription terminators, etc. (see Bitter
15 et al., 1987, Methods in Enzymol., 153:516-544).
          In addition, a host cell strain may be
chosen which modulates the expression of the inserted
sequences, or modifies and processes the gene product
in the specific fashion desired. Expression driven by
20 certain promoters can be elevated in the presence of
certain inducers, (e.g., zinc and cadmium ions for
metallothionein promoters). Therefore, expression of
the genetically engineered tau-protein or fragment
thereof may be controlled. This is important if the
25 protein product of the cloned foreign gene is lethal
to host cells. Furthermore, modifications (e.g.,
glycosylation) and processing (e.g., cleavage) of
protein products may be important for the function of
the protein. Different host cells have characteristic
30 and specific mechanisms for the post-translational
processing and modification of proteins. Appropriate
cell lines or host systems can be chosen to ensure the
correct modification and processing of the foreign
protein expressed.
35   The host cells which contain the tau-protein
or fragment thereof coding sequence and which express
the biologically active tau-protein or fragment

thereof gene product may be identified by at least four general approaches: (a) DNA-DNA hybridization; (b) the presence or absence of "marker" gene functions; (c) assessing the level of transcription as measured by expression of *tau*-protein mRNA transcripts in host cells; and (d) detection of *tau*-protein gene products as measured by immunoassays or by its biological activity.

In the first approach, the presence of the *tau*-protein or fragment thereof coding sequence inserted in the expression vector can be detected by DNA-DNA hybridization using probes comprising nucleotide sequences that are homologous to the *tau*-protein coding sequence or particular portions thereof substantially as described recently (Goeddert *et al.*, 1988, *Proc. Natl. Acad. Sci. USA*, 85:4051-4055).

In the second approach, the recombinant expression vector/host system can be identified and selected based upon the presence or absence of certain "marker" gene functions (*e.g.*, thymidine kinase activity, resistance to antibiotics, resistance to methotrexate, transformation phenotype, occlusion body formation in baculovirus, *etc.*). For example, if the *tau*-protein or fragment thereof coding sequence is inserted within a marker gene sequence of the vector, recombinants containing the *tau*-protein or fragment thereof coding sequence can be identified by the absence of the marker gene function. Alternatively, a marker gene can be placed in tandem with the *tau*-protein or fragment thereof coding sequence under the control of the same or different promoter used to control the expression of the *tau* coding sequence. Expression of the marker in response to induction or selection indicates expression of the *tau*-protein coding sequence.

In the third approach, transcriptional activity for the *tau*-protein or fragment thereof

coding region can be assessed by hybridization assays.
For example, RNA can be isolated and analyzed by
Northern blot using a probe homologous to the *tau*-
protein or fragment thereof coding sequence or

5 particular portions thereof substantially as described
(Goeddert *et al.*, 1988, *Proc. Natl. Acad. Sci. USA*,
85:4051-4055). Alternatively, total nucleic acids of
the host cell may be extracted and assayed for
hybridization to such probes.

10   In the fourth approach, the expression of
the *tau*-protein or fragment thereof product can be
assessed immunologically, for example by Western
blots, immunoassays such as radioimmunoprecipitation,
enzyme-linked immunoassays and the like.

15   Once a recombinant that expresses a *tau*-
protein or fragment thereof is identified, the gene
product should be analyzed. This can be achieved by
assays based on the physical, immunological or
functional properties of the product.

20   A *tau*-protein or fragment thereof should be
immunoreactive whether it results from the expression
of the entire gene sequence, a portion of the gene
sequence or from two or more gene sequences which are
ligated to direct the production of chimeric proteins.

25 This reactivity may be demonstrated by standard
immunological techniques, such as
radioimmunoprecipitation, radioimmune competition, or
immunoblots.

30   5.2 GENERATION OF ANTIBODIES THAT
                 DEFINE *TAU*-PEPTIDES
            Various procedures known in the art may be
used for the production of antibodies to epitopes of
the tau-protein. Such antibodies include but are not

35 limited to polyclonal, monclonal, chimeric, single
chain, Fab fragments and an Fab expression library.
For the production of antibodies, various host animals

may be immunized by injection with a particular *tau*-protein, or a synthetic *tau*-peptide, including but not limited to rabbits, mice, rats, *etc.* Various adjuvants may be used to increase the immunological

5 response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole

10 limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *corynebacterium parvum.*

Monoclonal antibodies to peptides of *tau* may be prepared by using any technique which provides for

15 the production of antibody molecules by continuous cell lines in culture. These include but are not limited to the hybridoma technique originally described by Kohler and Milstein, (*Nature*, 1975, 256:495-497), the more recent human B-cell hybridoma

20 technique (Kosbor *et al.*, 1983, *Immunology Today*, 4:72) and the EBV-hybridoma technique (Cole *et al.*, 1985, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc., pp. 77-96). In an additional embodiment of the invention monoclonal antibodies

25 specific to *tau*-peptides may be produced in germ-free animals utilizing recent technology (PCT/US90/02545). According to the invention, human antibodies may be used and can be obtained by using human hybridomas (Cote at al., 1983, *Proc. Natl. Acad. Sci.*, **80**:2026-

30 2030) or by transforming human B cells with EBV virus *in vitro* (Cole *et al.*, 1985, in, *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, pp. 77-96). In fact, according to the invention, techniques developed for the production of "chimeric antibodies" (Morrison

35 *et al.*, 1984, *Proc. Natl. Acad. Sci.*, **81**:6851-6855; Neuberger *et al.*, 1984, *Nature*, **312**:604-608; Takeda *et al.*, 1985, *Nature*, **314**:452-454) by splicing the genes

sample to be tested is brought into contact with the
bound molecule. After a suitable period of
incubation, for a period of time sufficient to allow
formation of an antibody-antigen binary complex, a
5 second antibody, labelled with a reporter molecule
capable of inducing a detectable signal, is then added
and incubation is continued allowing sufficient time
for binding with the antigen at a different site and
the formation of a ternary complex of antibody-
10 antigen-labeled antibody. Any unreacted material is
washed away, and the presence of the antigen is
determined by observation of a signal, which may be
quantitated by comparison with a control sample
containing known amounts of antigen. Variations on
15 the forward sandwich assay include the simultaneous
assay, in which both sample and antibody are added
simultaneously to the bound antibody, or a reverse
sandwich assay in which the labelled antibody and
sample to be tested are first combined, incubated and
20 added to the unlabelled surface bound antibody. These
techniques are well known to those skilled in the art,
and the possibility of minor variations will be
readily apparent. As used herein, "sandwich assay" is
intended to encompass all variations on the basic two-
25 site technique.
       For the sandwich assays of the present
invention, the only limiting factor is that both
antibodies have different binding specificities for
the tau-peptide. Thus, a number of possible
30 combinations are possible.
       As a more specific example, in a typical
forward sandwich assay, a primary antibody is either
covalently or passively bound to a solid support. The
solid surface is usually glass or a polymer, the most
35 commonly used polymers being cellulose,
polyacrylamide, nylon, polystyrene, polyvinylchloride
or polypropylene. The solid supports may be in the

form of tubes, beads, discs or microplates, or any
other surfaces suitable for conducting an immunoassay.
The binding processes are well known in the art.
Following binding, the solid phase-antibody complex is
5   washed in preparation for the test sample. An aliquot
of the body fluid containing the tau-peptide to be
tested is then added to the solid phase complex and
incubated at 25°C for a period of time sufficient to
allow binding of any tau-peptide present to the
10 antibody specific for tau-peptide. The second
antibody is then added to the solid phase complex and
incubated at 25°C for an additional period of time
sufficient to allow the second antibody to bind to the
primary antibody-antigen solid phase complex. The
15 second antibody is linked to a reporter molecule, the
visible signal of which is used to indicate the
binding of the second antibody to any antigen in the
sample. By "reporter molecule", as used in the
present specification is meant a molecule which by its
20 chemical nature, provides an analytically detectable
signal which allows the detection of antigen-bound
antibody. Detection must be at least relatively
quantifiable, to allow determination of the amount of
antigen in the sample, this may be calculated in
25 absolute terms, or may be done in comparison with a
standard (or series of standards) containing a known
normal level of antigen.
       The most commonly used reporter molecules in
this type of assay are either enzymes or fluorophores.
30 In the case of an enzyme immunoassay an enzyme is
conjugated to the second antibody, often by means of
glutaraldehyde or periodate. As will be readily
recognized, however, a wide variety of different
conjugation techniques exist, which are well known to
35 the skilled artisan. Commonly used enzymes include
horseradish peroxidase, glucose oxidase, β-
galactosidase and alkaline phosphatase, among others.

The substrates to be used with the specific enzymes
are generally chosen for the production, upon
hydrolysis by the corresponding enzyme, of a
detectable color change. For example, p-nitrophenyl
5 phosphate is suitable for use with alkaline
phosphatase conjugates; for peroxidase conjugates,
1,2-phenylenediamine or toluidine are commonly used.
It is also possible to employ fluorogenic substrates,
which yield a fluorescent product rather than the
10 chromogenic substrates noted above. In all cases, the
enzyme-labelled antibody is added to the first
antibody-tau-peptide complex and allowed to bind to
the complex, then the excess reagent is washed away.
A solution containing the appropriate substrate is
15 then added to the tertiary complex of antibody-
antigen-labelled antibody. The substrate reacts with
the enzyme linked to the second antibody, giving a
qualitative visual signal, which may be further
quantitated, usually spectrophotometrically, to give
20 an evaluation of the amount of antigen which is
present in the serum sample.

Alternately, fluorescent compounds, such as
fluorescein or rhodamine, may be chemically coupled to
antibodies without altering their binding capacity.
25 When activated by illumination with light of a
particular wavelength, the fluorochrome-labelled
antibody absorbs the light energy, inducing a state of
excitability in the molecule, followed by emission of
the light at a characteristic longer wavelength. The
30 emission appears as a characteristic color visually
detectable with a light microscope. As in the enzyme
immunoassay (EIA), the fluorescent-labelled antibody
is allowed to bind to the first antibody-tau-peptide
complex. After washing the unbound reagent, the
35 remaining ternary complex is then exposed to light of
the appropriate wavelength, and the fluorescence
observed indicates the presence of the antigen.

Immunofluorescence and EIA techniques are both very
well established in the art and are particularly
preferred for the present method. However, other
reporter molecules, such as radioisotopes,
5 chemiluminescent or bioluminescent molecules may also
be employed. It will be readily apparent to the
skilled artisan how to vary the procedure to suit the
required use.

        Alternatively, the sample to be tested
10 either human blood or spinal fluid containing the tau-
peptide may be used in a single site immunoassay
wherein it is adhered to a solid substrate either
covalently or noncovalently. An unlabeled anti-tau-
peptide antibody is brought into contact with the
15 sample bound on the solid substrate. After a suitable
period of incubation, for a period of time sufficient
to allow formation of an antibody-antigen binary
complex a second antibody, labelled with a reporter
molecule capable of inducing a detectable signal, is
20 then added and incubation is continued allowing
sufficient time for the formation of a ternary complex
of antigen-antibody-labeled antibody. For the single
site immunassay, the second antibody may be a general
antibody (i.e., zenogeneic antibody to immunoglobulin,
25 particularly anti-(IgM and IgG) linked to a reporter
molecule) that is capable of binding an antibody that
is specific for the tau-peptide of interest.

        Alternatively, the purified tau-proteins or
fragments thereof, described in Section 5.1, supra,
30 may be used in a diagnostic assay to detect
circulating anti-tau-peptide autoantibodies wherein
they are adhered to a solid support either covalently
or non-covalently. The sample, either human blood or
spinal fluid diluted in an appropriately buffered
35 medium, is added to the solid support and a sufficient
time is allowed for complex formation between the tau-
proteins or fragments thereof and any cognate

autoantibodies in the sample. The supernatant is
removed and the solid support is washed to remove any
non-specifically bound proteins. To the complex may
be added xenogeneic antisera to human immunoglobulin,
5 particularly anti-(human Ig M and Ig G) in an
appropriately buffered medium. The xenogeneic
antisera will normally be labeled with a reporter
molecule as described above for the forward sandwich
assay. Instead of xenogeneic antisera, proteins
10 specific for the immune complex may be employed, e.g.,
S. aureus protein A. The label may then be detected
as described above for the forward sandwich assay.

6. EXAMPLE: PRODUCTION AND PURIFICATION OF IMMUNOGENS FOR ANTIBODY PRODUCTION

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6.1. PURIFICATION OF MICROTUBULES FROM BOVINE BRAIN BY THE CYCLE PROCEDURE AS A SOURCE OF NATIVE, PHYSIOLOGICALLY ACTIVE MICROTUBULE ASSOCIATED PROTEINS

The cycle purification of microtubules from
20 bovine brain was performed in the presence of
guanosine triphosphate and low concentrations of
magnesium ions in order to obtain high ratios of
microtubule associated proteins to tubulin. This
cycle-purification was carried out in five separate
25 steps according to a modification of the method
described by Asnes & Wilson, (1979, Anal. Biochem.,
98:64-73).

Step (1) Transport & Dissection: Eight
bovine brains (approximately 350 gram (g) each) were
30 removed, cut into pieces, placed in ice-cold transport
buffer [20 mM phosphoric acid (adjusted to pH 6.75
with 2M NaOH) 100 mM sodium glutamate, 300 mM sucrose]
containing ice cubes made from transport buffer and
transported in an ice bucket from the abattoir to the
35 laboratory. The meninges, superficial blood vessels
and the majority of the white matter was removed and

discarded. The remaining brain tissue (approximately 1200 g) was drained and weighed.

Step (2) <u>Homogenization</u>: The selected brain tissue was dispersed using two separate maneuvers sequentially. The weighed brain tissue from step (1) was passed once through a domestic electric mincer and added to homogenization buffer [200 mM phosphoric acid (adjusted to pH 6.75 with 2 M NaOH), 100 mM sodium glutamate, 300 mM sucrose, 4 mM β-mercaptoethanol, 0.3 mM phenylmethanesulphonylfluoride (PMSF) (stock solution, 100 mM in dimethylformamide (DMF)), 0.3 mM Nα-p-tosyl-1-lysine chloromethylketone (TLCK) (prepared in PMSF stock solution), 50 μg/ml leupeptin, few crystals of catalase] (at 0°C and at a concentration of 1.5 ml buffer / g tissue). The material was dispersed by gentle stirring with a glass rod. The resulting suspension was then homogenized in three batches with two complete strokes (up and down twice for each batch separately) of a motor driven 1 liter (L) glass (2.2 inch diameter x 18 inch long) Potter - Elvehjem homogenizer fitted with a 2 inch long teflon pestle (0.005 inch clearance) at 0°C.

Step (3) <u>First Cycle</u>: Approximately 3000 ml of the homogenate was added to twelve 250 ml plastic centrifuge tubes and centrifuged at 27,880 g (13,000 rpm in two Sorval RC5B preparative centrifuges containing two GSA rotors precooled to 3°C) for 53 minutes at 0-4°C. The supernatants were carefully removed, combined and cofactor buffer [2.5 mM GTP, .50 mM MgCl$_2$, 1.0 mM EGTA (10 x stock solution adjusted to pH 6.75 at 0°C with NaOH)] prepared in homogenization buffer was added. The solution was mixed and then approximately 1L portions together with a glass thermometer was placed in separate polyethene sleeves (10 inch x 14 inch) and heat sealed. The loaded sleeves were gently agitated in a 50°C water bath until the temperature of their contents reached 30°C

whereupon they were immediately transferred to a 30°C
water bath where the incubation continued without
further agitation for the remainder of a total period
of 30 minutes. Assembly was followed viscometrically
5 using calibrated 100-size Cannon Ubbleohde glass
viscometers. Approximately 2400 ml of the assembled
extract was transferred to 12 clean 250 ml plastic
centrifuge jars and centrifuged at 27,880 g (13,000
rpm in two Sorval RC5B preparative centrifuges
10 containing two Sorval GSA rotors prewarmed to 30°C)
for 42 minutes at 30°C. The supernatant was carefully
removed with an aspirator and its volume was measured,
recorded and discarded.

    Step (4) Second Cycle: The final pellet from
15 the first cycle was resuspended in ice-cold
homogenization buffer without sucrose (10% of volume
of the supernatant from the first cycle) using a
precooled small Dounce homogenizer. Approximately
220 ml of the resuspended pellet was incubated at 0°C
20 for 30 minutes, added to six 36 ml plastic centrifuge
tubes, and centrifuged at 39,000 g (14,627 rpm in a
Sorval OTD centrifuge containing a Sorval AH-627 rotor
precooled to 3°C) for 40 minutes at 0 to 4°C. The
supernatant was carefully transferred to a 1L glass
25 conical flask and cofactor buffer was added. The
solution was mixed and gently swirled in the 50°C
water bath until the temperature reached 30°C. The
flask was then immediately transferred to a 30°C water
bath where the incubation continued without further
30 agitation for a total period of only 10 minutes. The
extract was transferred to six 36 ml plastic
centrifuge tubes and centrifuged at 39,000 g (14,726
rpm in a Sorval OTD ultracentrifuge containing a
Sorval AH-627 rotor) for 30 minutes at 30°C. The
35 supernatant was removed carefully with an aspirator
and its volume was measured and discarded.

Step (5) <u>Third Cycle</u> - The final pellet from
the second cycle was resuspended in ice-cold
homogenization buffer without sucrose (40% of the
volume of the supernatant from the second cycle) using
5   a clean precooled small Dounce homogenizer.
Approximately 90 ml of resuspended pellet was
incubated at 0°C for 30 minutes, transferred to three
36 ml tubes, and centrifuged at 39,000 g (14,726 rpm
in a Sorval OTD ultracentrifuge containing a Sorval
10 AH-627 rotor with 36 ml buckets) for 40 minutes at 0
to 4°C. Approximately 80 ml of supernatant was
carefully transferred to a 500 ml glass conical flask
and cofactor buffer was added. The solution was mixed
and gently swirled in the 50°C water bath until the
15 temperature of the contents of the flask reached 30°C.
Then the flask was immediately transferred to the 30°C
water bath where the incubation continued without
further agitation for a total period of 20 minutes.
The warm extract was transferred to six 17 ml plastic
20 centrifuge tubes and centrifuged at 39,000 g (Sorval
PTD centrifuge containing a Sorval AH-627 rotor with
17 ml buckets) for 40 minutes at 30°C. The
supernatant was removed carefully with an aspirator
and discarded. Each tube contained a pellet of
25 approximately 140 mg of microtubule (MT) proteins
(approximately 50% tubulin and 50% MAPS) and was
stored at -70°C.

6.2. ISOLATION OF THE *TAU* CLASS OF MICROTUBULE ASSOCIATED PROTEINS FROM CYCLE PURIFIED MICROTUBULES

30

The total MAPS fraction of cycle purified
microtubules was separated from tubulin by a
modification of the method of Weingarten, (1975, *Proc.*
35 *Natl. Acad. Sci. USA*, 72:1858-1862). A pellet of
microtubules from the cycle purification method was
resuspended using a small Dounce homogenizer in MES

buffer (0.1 M MES; 1 mM EGTA; 0.5 mM MgCl$_2$ and 1 mM β-
mercaptoethanol) so that the resulting solution had an
absorbance at 280 nm of 2.0 - 2.5 and then
depolymerized by incubation for 30 min at 0°C. The
5   resulting solution was centrifuged at 100,000 g for 1
hour and the pellet discarded. The supernatant was
filtered through a 0.2 μ milipore filter and applied
to a phosphocellulose column (1.1 cm diameter x 2.6 cm
length, 2.5 ml bed volume) that had previously been
10 equilibrated in MES buffer. Tubulin was eluted with
MES buffer in the void volume of the column and when
the absorbance of the eluate had returned to the
baseline value, the total MAPS fraction was eluted
(either 0.1 or 0.2 ml fractions) with MES buffer
15 containing 1.0 M NaCl and collected as a single
complex peak of UV-absorbing material that contained
multiple proteins.

The total MAPS fraction eluted from the
phosphocellulose column was immediately desalted using
20 the rapid Sephadex G-25 centrifugation method of
Penefsky (*J. Biol. Chem.*, 1977, **252**:2891-2899). The
desalted total MAPS fraction was stirred at 0° C and
solid ammonium sulphate added until its conentration
reached 35% (w/v). The stirring was continued for 30
25 minutes and then the suspension was centrifuged at
20,000 g for 15 minutes. The supernatant was removed
and again stirred at 0° C and more solid ammonium
sulphate added until its concentration reached 45%
(w/v).   After a further 15 minute stirring the
30 suspension was again centrifuged at 20,000 g for 15
minutes and the supernatant discarded. The pellet
from this last centrifugation step was dissolved in
Mes buffer and applied to a column of hydroxyapatite
(1 cm dia x 10 cm length) and eluted with Mes buffer.
35 The fractions containing only four closely spaced
peptides of apparent Mr 45,000 to 65,000 by SDS-PAGE
were combined. This total eluted *tau* fraction was

reduced to a volume of 1.0 ml with a sartorius collodion membrane operated in water under reduced pressure, according to the instructions of the manufacturer, and then divided into 0.1 ml portions in

5   0.5 ml plastic minifuge tubes and stored at -20°C.

The invention is not limited to the embodiments described herein and may be modified or varied without departing from the scope of the invention.

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WHAT IS CLAIMED IS:

1.   A method for diagnosing, subtyping or monitoring Alzheimer's disease in a patient,
5 comprising assaying a sample of body fluid obtained from the patient for the presence of a *tau*-peptide using either an anti-*tau*-peptide antibody or an anti-*tau*-peptide Fab fragment, wherein further the *tau*-peptide is derived from proteolytic action on
10 neurofibrillary tangles resulting in fragments from the amino terminal 200 amino acids or carboxy terminal 50 amino acids.

2.   A method for diagnosing, subtyping or
15 monitoring Alzheimer's disease in a patient, comprising assaying a sample of body fluid obtained from the patient for the presence of an anti-*tau*-peptide autoantibody.

20   3.   The method for diagnosing, subtyping or monitoring Alzheimer's disease according to claims 1 or 2, wherein the assay is a radioimmunoassay, an enzyme-linked immunosorbant assay, a sandwich assay, a precipitin reaction, a gel immunodiffusion assay, an
25 agglutination assay, a fluorescent immunoassay, a protein A immunoassay or an immunoelectrophoresis assay.

4.   A kit for detecting the presence of a *tau*-
30 peptide in a sample of body fluid comprising an anti-*tau*-peptide antibody or an anti-*tau*-peptide Fab fragment and means for detecting the binding of the *tau*-peptide with the antibody or fragment.

35
5.   A kit for detecting the presence of an anti-*tau*-peptide autoantibody in a sample of body fluid

comprising a *tau*-peptide and means for detecting the
binding of the autoantibody with the *tau*-peptide.

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-37 CCGCCTCTGTCGACTATCAGGTGAACTTTGAACCAGG

  1  Met Ala Glu Pro Arg Gln Glu Phe Glu Val Met Glu Asp His Ala Gly Thr Tyr Gly Leu Gly Asp Arg Lys Asp Gln Gly Gly Tyr Thr
  1  ATG GCT GAG CCC CGC CAG GAG TTC GAA GTG ATG GAA GAT CAC GCT GGG ACG TAC GGG CTT GGG GAC AGG AAA GAT CAG GGC TAC ACC

 31  Met His Gln Asp Glu Gly Asp Thr Asp Ala Gly Leu Lys Ala Glu Glu Ala Gly Ile Gly Asp Thr Pro Ser Leu Glu Asp Glu Ala
 91  ATG CAC CAA GAC GAG GGT GAC ACG GAC GCT GGC CTG AAA GCT GAA GAA GCA GGC ATT GGA GAC ACC CCC AGC CTG GAA GAC GAA GCT

 61  Ala Gly His Val Thr Gln Ala Arg Met Val Ser Lys Ser Lys Asp Gly Thr Gly Ser Asp Asp Lys Lys Ala Lys Gly Ala Asp Gly Lys
101  GCT GGT CAC GTG ACC CAA GCT CGC ATG GTC AGT AAA AGC AAA GAC GGC ACT GGA AGC GAT GAC AAG AAA GCC AAG GGG GCT GAT GGT AAA

 91  Thr Lys Ile Ala Thr Pro Arg Gly Ala Ala Pro Gly Gln Lys Gly Gln Ala Asn Ala Thr Arg Ile Pro Ala Lys Thr Pro Pro Ala
271  ACG AAG ATC GCC ACA CCG CGG GGA GCA GCC CCT CCA GGA CAG AAG GGC CAG GCC AAC GCC ACC AGG ATT CCA GCA AAA ACC CCG CCC GCT

121  Pro Lys Thr Pro Pro Ser Ser Gly Glu Pro Pro Lys Ser Gly Asp Arg Ser Gly Tyr Ser Ser Pro Gly Thr Pro Gly Ser
361  CCA AAG ACA CCA CCC AGC TCT GGT GAA CCT CCA AAG TCA GGG GAT CGC AGC GGC TAC AGC AGC CCC GGC ACT CCC GGC AGC

151  Arg Ser Arg Thr Pro Ser Leu Pro Thr Pro Pro Thr Arg Glu Pro Lys Lys Val Ala Val Val Arg Thr Pro Pro Lys Ser Pro Ser Ser
451  CGC TCC CGC ACC CCG TCC CTT CCA ACC CCA CCA ACC CGG GAG CCC AAG AAG GTG GCA GTG GTC CGT ACT CCA CCC AAG TCG CCG TCT TCC

181  Ala Lys Ser Arg Leu Gln Thr Ala Pro Val Pro Met Pro Asp Leu Lys Asn Val Lys Ser Lys Ile Gly Ser Thr Glu Asn Leu Lys His
541  GCC AAG AGC CGC CTG CAG ACA GCC CCC GTG CCC ATG CCC GAC CTG AAG AAT GTC AAG TCC AAG ATC GGC TCC ACT GAG AAT CTG AAG CAC
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FIG.1

211 Gln Pro Gly Gly Lys Val Gln Ile Val Tyr Lys Pro Val Asp Leu Ser Lys Val Thr Ser Lys Cys Gly Ser Leu Gly Asn Ile His
631 CAG CCG GGA GGC GGG AAG GTG CAA ATA GTC TAC AAA CCA GTT GAC CTG AGC AAG GTG ACC TCC AAG TGT GGC TCA TTA GGC AAC ATC CAT

241 His Lys Pro Gly Gly Gln Val Glu Val Lys Ser Glu Lys Leu Asp Phe Lys Asp Arg Val Gln Ser Lys Ile Gly Ser Leu Asp Asn
721 CAT AAA CCA CCA GGT GGC CAG GTA GTG GAA TCT GAG AAA CTT GAC TTC AAG GAC AGA GTC CAG TCG AAG ATT GGG TCC CTG GAC AAT

271 Ile Thr His Val Pro Gly Gly Gly Asn Lys Lys Ile Glu Thr His Lys Leu Thr Phe Arg Glu Asn Ala Lys Thr Asp His Gly
811 ATC ACC CAC GTC CCT GGC GGA GGA AAT AAG AAG ATT GAA ACC CAC AAG CTG ACC TTC CGC GAG AAC GCC AAA ACA GAC CAC GGG

301 Ala Glu Ile Val Tyr Lys Ser Pro Val Val Ser Gly Asp Thr Ser Pro Arg His Leu Ser Asn Val Ser Ser Thr Gly Ser Ile Asp Met
901 GCG GAG ATC GTG TAC AAG TCG CCA GTG GTG TCT GGG GAC ACG TCT CCA CGG CAT CTC AGC AAT GTC TCC ACC GGC AGC ATC GAC ATG

331 Val Asp Ser Pro Gln Leu Ala Thr Leu Ala Asp Glu Val Ser Ala Ser Leu Ala Lys Gln Gly Leu ***
991 GTA GAC TCG CCC CAG CTC GCC ACG CTA GCT GAC GAG GTG TCT GCC TCC CTG GCC AAG CAG GGT TTG TGA TCAGGCCCTGG

FIG.1(CONT.)

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :Please See Extra Sheet.

US CL :435/7.92, 7, 808, 811; 436/ 518, 515, 516; 530/387.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.92, 7, 808, 811; 436/ 518, 515, 516; 530/387.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CAS, MEDLINE, APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	PROCEEDINGS OF NATIONAL ACADEMY OF SCIENCES, USA, VOLUME 83, ISSUED JULY 1986, I. GRUNDKE-IQBAL ET AL., "ABNORMAL PHOSPHORYLATION OF THE MICROTUBULE-ASSOCIATED PROTEIN Г (TAU) IN ALZHEIMER CYTOSKELETAL PATHOLOGY", PAGES 4913-4917, SEE ENTIRE DOCUMENT.	1-5
Y	JOURNAL OF NEUROSCIENCE RESEARCH, VOLUME 25, ISSUED 1990, H. KSIEZAK-REDING ET AL., " "MAPPING OF THE ALZ-50 EPITOPE IN MICROTUBULE-ASSOCIATED PROTEINS TAU", PAGES 412-419, ENTIRE DOCUMENT.	1-5
Y	LANCET, ISSUED 06 JULY 1985, P. D. MEHTA ET AL., "PAIRED HELICAL FILAMENT ANTIGEN IN CSF", PAGE 35. SEE ENTIRE DOCUMENT.	1-5
Y	AMERICAN JOURNAL OF PATHOLOGY, VOLUME 126, NO. 1, ISSUED JANUARY 1987, SHU-HUI YEN ET AL., "ALZHEIMER'S NEUROFIBRILLARY TANGLES CONTAIN UNIQUE EPITOPES AND EPITOPES IN COMMON WITH THE HEAT-STABLE MICROTUBULE ASSOCIATED PROTEINS TAU AND MAP2", PAGES 81-91, ENTIRE DOCUMENT.	1-5

☐ Further documents are listed in the continuation of Box C.　　☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O"	document referring to an oral disclosure, use, exhibition or other means	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
27 August 1992	1 0 SEP 1992

Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer BETTY J. HAYDEN, PH.D.
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Form PCT/ISA/210 (second sheet)(July 1992)*

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (5): G01N 33/53; A61K 35/12